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Neuronal Nitric Oxide Synthase Signaling in the Heart Is Regulated by the Sarcolemmal Calcium Pump 4b

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Background—Neuronal nitric oxide synthase (nNOS) has recently been shown to be a major regulator of cardiac contractility. In a cellular system, we have previously shown that nNOS is regulated by the isoform 4b of plasma membrane calcium/calmodulin-dependent ATPase (PMCA4b) through direct interaction mediated by a PDZ domain (PSD 95, Drosophila Discs large protein and Zona occludens-1) on nNOS and a cognate ligand on PMCA4b. It remains unknown, however, whether this interaction has physiological relevance in the heart *in vivo*.

Methods and Results—We generated 2 strains of transgenic mice overexpressing either human PMCA4b or PMCA ct120 in the heart. PMCA ct120 is a highly active mutant form of the pump that does not interact with or modulate nNOS function. Calcium was extruded normally from PMCA4b-overexpressing cardiomyocytes, but *in vivo*, overexpression of PMCA4b reduced the β -adrenergic contractile response. This attenuated response was not observed in ct120 transgenic mice. Treatment with a specific nNOS inhibitor (*N* ω -propyl-L-arginine) reduced the β -adrenergic response in wild-type and ct120 transgenic mice to levels comparable to those of PMCA4b transgenic animals. No differences in lusitropic response were observed in either transgenic strain compared with wild-type littermates.

Conclusions—These data demonstrate the physiological relevance of the interaction between PMCA4b and nNOS and suggests its signaling role in the heart. (*Circulation*. 2007;115:483-492.)

Key Words: signal transduction ■ nitric oxide synthase ■ calcium ■ contractility

Neuronal nitric oxide synthase (nNOS) plays an important role in a number of essential aspects of cardiac physiology. Evidence has shown that nNOS is involved in regulating cardiac contractility,^{1,2} calcium cycle,^{3,4} and redox equilibrium.⁵ In general, nitric oxide synthase (NOS) activity is regulated by a variety of mechanisms, including cytoskeletal dynamics⁶ and protein-protein interactions.⁷ For example, in skeletal muscle, nNOS has been demonstrated to interact with syntrophin,⁸ a dystrophin-associated cytoskeletal protein, and in neuronal cells, it has been described that CAPON (carboxy-terminal PDZ [PSD 95, Drosophila Discs large protein and Zona occludens-1] ligand of nNOS) competes with PSD95 for interaction with nNOS and may in turn indirectly restrict NO generation.⁹

Clinical Perspective p 492

We have previously shown in an *in vitro* system (HEK293 cells) that the isoform 4b of the sarcolemmal calcium pump (also known as plasma membrane calcium/calmodulin-dependent calcium ATPase, or PMCA) is able to tightly

regulate nNOS. PMCA4b and nNOS form a PDZ domain-mediated interaction in which PMCA4b is likely to regulate nNOS activity by altering local calcium concentration.¹⁰ The physiological consequences of PMCA4b-mediated nNOS regulation in the heart remain unknown, however.

To address this question, we generated transgenic mice overexpressing either full-length human PMCA4b or the mutant form, PMCA ct120, both under the control of the myosin light chain (MLC2v) promoter. The PMCA ct120 lacks 120 amino acid residues at the COOH terminus, including the autoinhibitory (calmodulin binding) domain and the PDZ binding motif.¹¹ It has been shown that this mutant molecule is highly active as a calcium pump¹¹ but is unable to downregulate nNOS activity.¹⁰ Using these models, we demonstrated that PMCA4b regulates cardiac contractility *in vivo* through its interaction with nNOS.

Methods

Generation of Transgenic Mice

To generate mice overexpressing PMCA4b in the heart, human PMCA4b cDNA (a kind gift from Dr E. Strehler, Rochester, Minn)

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driven by the rat MLC2v promoter (a kind gift from Dr K. Chien and Genentech Inc, San Francisco, Calif) was microinjected into the pronuclei of single-cell embryos (C57Bl6×CBA; Manchester, UK) by standard techniques. The pMMct120 plasmid, which contained PMCA ct120 cDNA, was a gift from Dr E. Strehler. The MLC2v-ct120 construct was generated by replacing the ≈0.8-kb *NsiI-KpnI* fragment of the MLC2v-PMCA4b with the ≈0.4-kb *NsiI-KpnI* fragment of ct120. The transgene was excised from the vector and microinjected into the same strain of embryo as above. Transgenic mice were detected by polymerase chain reaction (PCR) with the following primers: forward 5'-GGCTCCCTGAGTGACTCCC-3'; reverse 5'-CCTGATGACGGTGCTCATTG-3'. Transgenic founders were mated with wild-type littermates to establish the line.

All animal experimentations were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. Hemodynamic and echocardiography analyses were conducted at 4 to 5 months of age. Age-matched wild-type littermates from PMCA4b transgenic and ct120 transgenic lines were used as controls. See the online Data Supplement, Methods section, for details of echocardiography, left ventricular hemodynamic analysis, response to NOS inhibition, and cardiac hypertrophy model.

Analysis of Transgene Expression

Transgene expression was determined by Western blot and reverse transcription (RT)-PCR. For Western blot, hearts of transgenic mice and wild-type controls were homogenized in radioimmunoprecipitation assay buffer (1X PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 20 μmol/L PMSF, 500 ng/mL leupeptin, 1 μg/mL aprotinin, and 500 ng/mL pepstatin). Lysates (30 μg) were electrophoresed in 8% SDS-PAGE gel and transferred to nitrocellulose membrane. We used polyclonal anti-PMCA4 antibody (Swant, Bellinzona, Switzerland) to determine the level of overexpression. For RT-PCR, total RNA from the heart was isolated with Trizol reagent (Invitrogen, Carlsbad, Calif). Total RNA (2 μg) was reverse transcribed with MMLV-RT (Promega, Madison, Wis). The PMCA4b and ct120 transgenes were PCR amplified with the following primers: forward 5'-AGCCCCTACGGAATCTTCGTT-3' and reverse 5'-CTCATTAAGGCATTCCACCACTG-3', which were designed to amplify the COOH terminus of the transgene, producing an ≈1.15-kb PMCA4b fragment and an ≈0.78-kb ct120 fragment.

Real-Time Quantitative RT-PCR

Real-time RT-PCR was performed with QuantiTect SYBR-green RT-PCR kit (Qiagen, Valencia, Calif) with conditions recommended by the manufacturer. Relative standard curves were generated for each gene tested with the available protocol (Applied Biosystems, Foster City, Calif). The level of GAPDH mRNA was used as the loading control. Primers to detect mPMCA4, nNOS, and GAPDH were obtained from Qiagen (QuantiTect Primer Assay kit).

Single Cardiac Myocyte Studies

Mice (3 months of age) were euthanized by intraperitoneal injection of pentobarbitone (200 mg/kg). The hearts were removed rapidly and perfused via the aorta with a nominally Ca²⁺-free solution, and single ventricular myocytes were isolated by a modified collagenase and protease digestion technique.¹² Changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) were measured with Fluo-3AM at 37°C as described previously.¹²

Immunoprecipitation and Western Blot

Hearts were homogenized in radioimmunoprecipitation assay buffer, and protein content was determined with a BCA protein assay reagent kit (Pierce, Rockford, Ill). Protein lysates were immunoprecipitated with anti-nNOS (Affinity Bioreagents, Golden, Colo) or anti-luciferase antibody (Promega) by methods described previously.¹³ Western blots were conducted by separating equal amounts of protein in an SDS-PAGE system; primary antibodies used were polyclonal anti-PMCA4 antibody (Swant), anti-Na⁺/Ca²⁺ exchanger antibody (Swant), anti-sarcoplasmic reticulum Ca²⁺-ATPase 2a

antibody (Affinity Bioreagents), anti-phospholamban antibody (Upstate, Lake Placid, NY), anti-dihydropteridine reductase-α antibody (Affinity Bioreagents), and anti-nNOS antibody (Affinity Bioreagents). GAPDH expression (detected with antibody from Abcam, Cambridge, United Kingdom) was used for loading control. Levels of expression were determined with Alpha Imager software (Alpha Innotech, San Leandro, Calif).

Data Analysis

Data are expressed as mean±SEM and analyzed with the Student *t* test, 1-way ANOVA, or 2-factor ANOVA, followed by post hoc multiple comparison test where appropriate. The criterion of statistical significance was *P*<0.05.

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Effects of Overexpressing PMCA4 on Cellular Calcium Dynamics

We generated transgenic mice with overexpression of PMCA4b targeted to cardiomyocytes using a myosin light chain (MLC2v) promoter (Figure 1A). Levels of overexpression were assessed with anti-PMCA4 polyclonal antibody, which also recognizes endogenous mouse PMCA4. We produced 2 independent lines of transgenic mice with moderate levels (≈1.5-fold) of transgene overexpression in the heart (Figure 1B).

To test whether overexpression of PMCA4b altered intracellular calcium dynamics, we examined intracellular Ca²⁺ transients during depolarization in isolated cardiomyocytes (Figure 1C). The time constant of [Ca²⁺]_i decay was not different between PMCA4b-overexpressing myocytes and wild-type controls, either basally or after treatment with isoproterenol (Figure 1D), thereby confirming the absence of a direct effect of PMCA4b on diastolic calcium removal during excitation-contraction coupling. However, a slight reduction of calcium amplitude (ΔF/F₀) in response to isoproterenol treatment was observed in cardiomyocytes overexpressing PMCA4b, although the difference did not reach statistical significance (*P*=0.08; Figure 1E).

PMCA4b Overexpression Reduced the β-Adrenergic Inotropic Response

In vivo cardiac function was assessed with echocardiographic and invasive hemodynamic analyses. Echocardiographic data indicated that PMCA4b-overexpressing mice showed normal chamber dimensions and wall thickness. Systolic and diastolic left ventricular function was evaluated from the pressure-volume loop data. No significant differences in any baseline hemodynamic parameters were found between PMCA4b transgenic mice and wild-type littermates (online Data Supplement, Table I).

To determine the effects of β-adrenergic stimulation on cardiac contractility, we analyzed the pressure-volume data after injection with isoproterenol 800 ng/kg body weight. Representative baseline and isoproterenol-induced pressure-volume loops are presented in Figure 2. End-systolic elastance (E_{es}), an indicator of cardiac contractility, was increased by 40±7% in wild-type mice. This response was significantly attenuated in mice overexpressing PMCA4b (E_{es} change

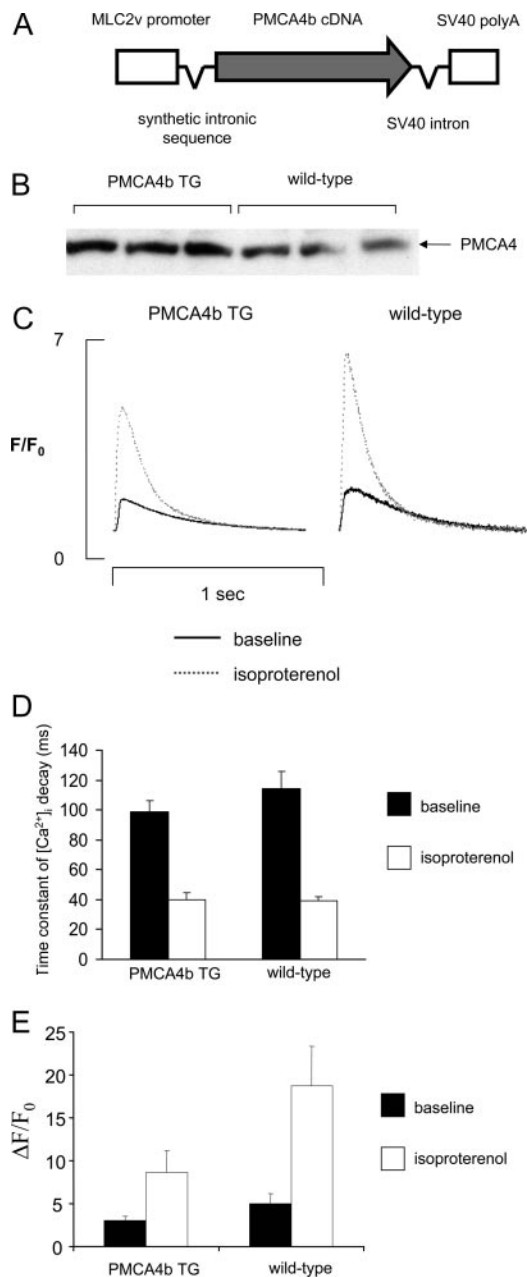


Figure 1. Overexpression of PMCA4b in the heart. A, Schematic representation of the transgenic vector MLC2v-PMCA4b. B, Western blots with polyclonal anti-PMCA4 (Swant) antibody in heart tissue of wild-type and PMCA4b-overexpressing mice (PMCA4b TG). C, Representative calcium transients of cardiomyocytes isolated from PMCA4b TG and wild-type littermate at baseline and after stimulation with isoproterenol 0.3 μ g/L. D, Time constant of rate decay of Ca^{2+} transients was not significantly different between PMCA4b TG and wild-type littermates ($n=5$ myocytes isolated from 3 independent animals in each group). E, The amplitude of Ca^{2+} transients ($\Delta F/F_0$) was not different at baseline, but in response to isoproterenol stimulation, cardiomyocytes isolated from PMCA4b TG mice displayed reduced $\Delta F/F_0$ compared with wild type.

11 \pm 5%, $P < 0.05$ versus wild type; Figure 2C). To verify this finding, we also compared the E_{es} change using multiple linear regression analysis with dummy variables.¹⁴ Consistently, a significant reduction in the E_{es} change was observed in PMCA4b-overexpressing mice ($P < 0.05$; Figure 2D). The relationship between dP/dt_{max} and end-diastolic volume (dP/dt_{max} -EDV) was also examined as another index of systolic function.

The dP/dt_{max} -EDV after isoproterenol injection was significantly reduced in PMCA4b transgenic mice compared with wild-type controls ($P < 0.05$; Figure 2E).

To determine the lusitropic responses to β -adrenergic stimulation, the time constant of left ventricular relaxation (τ) was analyzed. τ was not significantly different between transgenic animals and wild-type littermates after isoproterenol stimulation (Figure 2F). These results suggest that PMCA4 transgenic mice exhibit a blunted systolic but not diastolic response to β -adrenergic stimulation.

Overexpression of a Non-nNOS Binding Form of PMCA4 Did Not Affect Cardiac Contractility

PMCA4b interacts with nNOS through its PDZ-binding domain at the C-terminus region.¹⁰ A mutant active form of PMCA4 with deletion of 120 amino acids at the C-terminus region, known as PMCA4 ct120 (Figure 3A), does not interact with nNOS.¹⁰ To elucidate whether the interaction with nNOS is responsible for the attenuation of the β -adrenergic response in PMCA4b transgenic mice, we generated transgenic mice overexpressing PMCA4 ct120 in the heart. We used a similar overexpression cassette as was used to generate the PMCA4b transgenic line and replaced the cDNA coding region with the ct120 construct. RT-PCR analysis showed expression of the ct120 transgene in the heart, whereas Western blot analysis revealed that the expression level of the ct120 transgene was approximately at a level similar to that of the endogenous PMCA4 (Figure 3B). To test the interaction of transgenic proteins with nNOS, we immunoprecipitated heart extracts from PMCA4b and ct120 transgenic mice with an antibody specific for nNOS, followed by Western blot detection with anti-PMCA4 antibody. An irrelevant antibody (anti-luciferase) was used as a negative control for immunoprecipitation. We found an increased PMCA4-nNOS interaction in the PMCA4b transgenic mice compared with ct120 transgenic mice, as described in Figure 3C.

We then analyzed the cardiac phenotypes of 2 independent ct120 transgenic lines. Echocardiographic assessment showed no cardiac morphological abnormalities in these animals (online Data Supplement, Table II). The intracellular calcium dynamics in isolated myocytes were also examined, and we found that the rate constant of $[Ca^{2+}]_i$ decay was not different in ct120-overexpressing myocytes compared with wild-type controls either basally or after isoproterenol induction (data not shown).

Left ventricular catheterization revealed that ct120 transgenic mice exhibited normal cardiac function under basal conditions (Data Supplement, Table II). In contrast to PMCA4b transgenic mice, however, the ct120-overexpressing mice did not show reduced β -adrenergic inotropic responses, as indicated by the percentage increase of E_{es} and the dP/dt_{max} -EDV values (Figure 4A through 4E). Equally important, no difference in the lusitropic response to β -adrenergic stimulation was observed in ct120 transgenic mice, as indicated by the relaxation time constant values (Figure 4F).

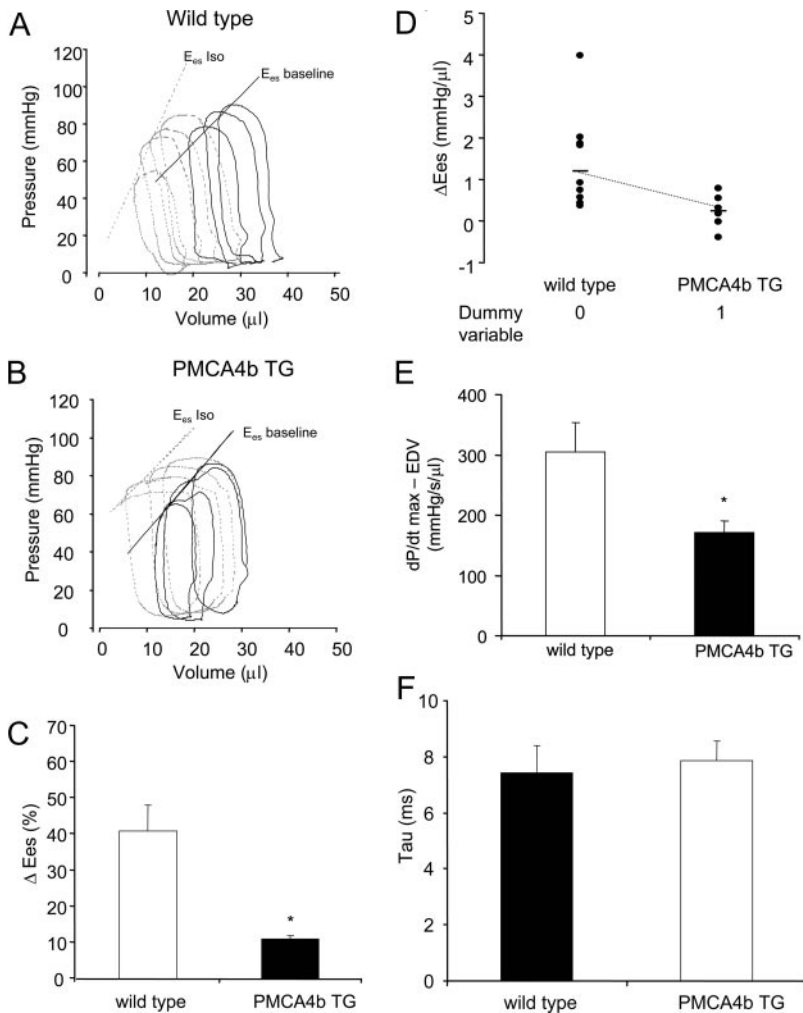


Figure 2. The β -adrenergic inotropic response was attenuated in mice overexpressing PMCA4b. Representative pressure-volume loops and E_{es} obtained by transient inferior cava vein occlusion at baseline (solid lines) and after isoproterenol (Iso; 800 ng/kg body weight; dashed lines) from (A) wild-type and (B) PMCA4b transgenic (TG) mice. Mice overexpressing PMCA4b have a reduced contractile response (indexed by the slope of E_{es} ; upward deflection signifies higher contractility) compared with wild-type controls. C, Summary of the E_{es} change (ΔE_{es}) in PMCA4b TG (n=6) and wild-type controls (n=12). * P <0.05 vs wild type. D, Regression analysis with dummy variables showed significant reduction of ΔE_{es} in PMCA4b TG mice. Each dot represents an individual animal, dotted line represents regression line (regression equation: $y = -0.972 + 1.21x$; $P < 0.05$). E, The $dP/dt_{max}-EDV$, another index of contractile response, was also reduced in PMCA4b TG mice. * P <0.05 vs wild type. F, The lusitropic response as indicated by relaxation time constant (τ) was not different between PMCA4b TG and wild-type controls.

Expression of Ca^{2+} Regulatory Genes

To investigate whether overexpression of PMCA4b and PMCA ct120 changed the expression levels of other major calcium transporters, we examined the protein levels of the Na/Ca exchanger, sarcoplasmic reticulum Ca^{2+} -ATPase 2a, phospholamban, and dihydropyridine reductase- α (L-type Ca^{2+} channel) in heart homogenates. Heart extracts from 5 animals in each group were subjected to Western blot analysis. Values shown in Figure 5A through 5H are levels of expression relative to wild-type control after normalization with GAPDH. There were no changes in expression levels of any proteins tested or in the sarcoplasmic reticulum Ca^{2+} -ATPase/phospholamban ratio (Figure 5I and 5J) in PMCA4b or ct120 transgenic mice.

Expression of nNOS and Endogenous PMCA4

Western blot and real-time RT-PCR were used to test whether transgenic overexpression of PMCA4b and PMCA ct120 modified the expression of nNOS in the heart. No differences in nNOS protein levels were observed between either transgenic line or wild-type littermates, as shown in Figure 5K and 5L. In keeping with that finding, quantitative RT-PCR analysis indicated that nNOS mRNA levels were not significantly different among PMCA4b-overexpressing mice (2.80 ± 0.12), ct120 transgenic mice (2.58 ± 1.29), and wild-type controls

(3.36 ± 1.88 ; values are picograms of mRNA per 1 mg of total RNA).

We also examined the levels of endogenous mPMCA4 expression in the heart. Real-time RT-PCR analysis indicated that there were no significant differences in endogenous PMCA4 expression among PMCA4b transgenic mice (166 ± 12), ct120 transgenic mice (212 ± 24), and wild-type littermates (195 ± 26 ; values are picograms of mRNA per 1 mg of total RNA).

PMCA4b-nNOS Interaction Was Responsible for Attenuation of the β -Adrenergic Response in PMCA4b Transgenic Mice

PMCA4b has previously been demonstrated to negatively regulate nNOS activity in vitro.¹⁰ To further test whether the PMCA4b-nNOS interaction was the mechanism responsible for the reduced β -adrenergic inotropic response in transgenic mice, we investigated the effect of NOS inhibition on cardiac contractility. We treated PMCA4b and ct120 transgenic and wild-type mice with the specific nNOS inhibitor N-propyl-L-arginine (L-nPA) and subsequently examined the effect of β -adrenergic stimulation. Figure 6A showed a subtle but nonsignificant increase in basal contractility in response to L-nPA. Treatment with L-nPA inhibited the isoproterenol-induced increase in E_{es} in wild-type and ct120 transgenic

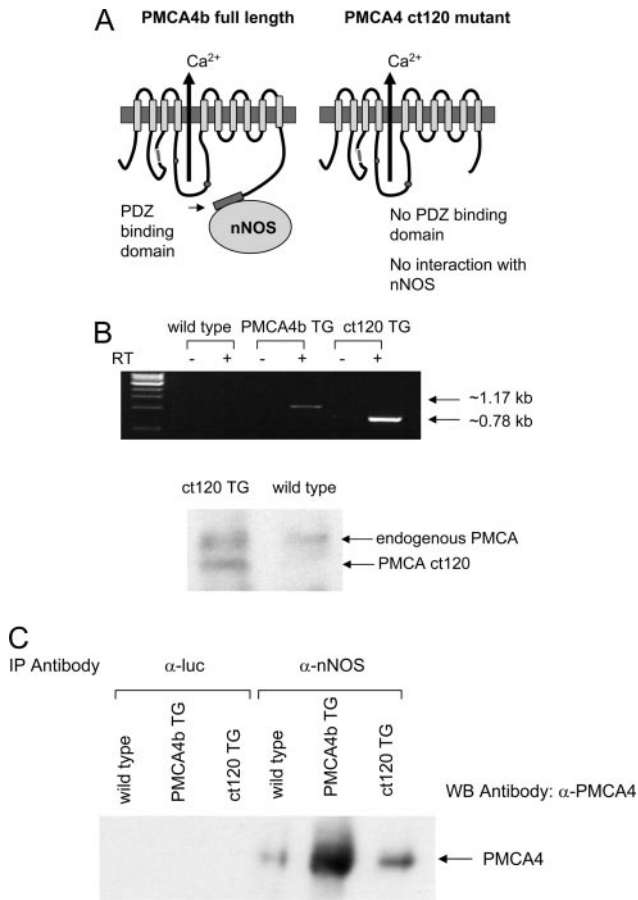


Figure 3. Overexpression of mutant form of PMCA4 ct120 in the heart. **A**, Schematic of mutant PMCA4b ct120. PMCA4b ct120 is active but lacks the PDZ binding domain at the COOH terminus and is unable to bind to nNOS. **B**, Expression of ct120 transgene in the heart was detected by RT-PCR and Western blot. Reverse-transcribed RNA (RT+) and non-reverse-transcribed RNA (RT-) isolated from ct120 transgenic (ct120 TG) and PMCA4b transgenic (PMCA4b TG) mice and wild-type controls were PCR amplified with primers specific for the COOH terminus of the transgenes (top panel). Western blot analysis (bottom panel) showed the expression of ct120 protein. **C**, Immunoprecipitation (IP) of heart extracts with anti-nNOS antibody (α-nNOS) followed by Western blot (WB) with anti-PMCA4 antibody showed increased PMCA4b-nNOS interaction in PMCA4b TG but not in ct120 TG mice. Immunoprecipitation with irrelevant antibody (α-Luc) showed no nonspecific binding of immunoprecipitating antibodies.

mice, however, but no significant effect was found in PMCA4b transgenic mice (Figure 6B and 6C).

To determine whether the observed changes in E_{es} were caused solely by nNOS inhibition or whether other NOS isoforms were involved, we further treated animals with the nonselective NOS inhibitor N^G-nitro-L-arginine methyl ester. No further decrease of E_{es} was observed after treatment with N^G-nitro-L-arginine methyl ester compared with L-nPA treatment, which suggests that the reduced cardiac contractile response to isoproterenol was the result of nNOS inhibition.

PMCA4b Transgenic Mice Displayed Higher Response to Hypertrophic Stimulus

On the basis of the finding that PMCA4b regulates the β-adrenergic inotropic response, we hypothesized that

PMCA4b may also be involved in pathological hypertrophy in response to long-term β-adrenergic stimulation. To model this condition, we treated PMCA4b transgenic, ct120 transgenic, and wild-type mice with isoproterenol for 7 days. With the dose of 10 mg/kg body weight per day, we found a nonsignificant increase of heart weight/body weight ratio compared with saline-treated controls in wild-type and ct120 transgenic mice. However, PMCA4b transgenic mice showed higher and statistically significant increases in heart weight/body weight ratio in response to a similar hypertrophic challenge (*P*<0.05; Figure 7A). Echocardiographic analysis suggested concentric remodeling in the PMCA4b transgenic mice, as indicated by a significant reduction in left ventricular end-diastolic diameter (Figure 7B).

Discussion

The present study was designed to investigate whether our previous finding in a cellular system (HEK293 cells) that PMCA4b regulates nNOS functions¹⁰ has relevance in the heart. We used a “gain of function” model to address this question, and the key finding was that PMCA4b regulates cardiac contractility through modulation of nNOS activity.

nNOS has recently been shown to be an important regulator of cardiac contractility in healthy^{2,4} and failing¹⁵ hearts, likely through its regulation of intracellular calcium dynamics. nNOS also regulates the β-adrenergic contractile response: mice deficient in nNOS displayed attenuated β-adrenergic inotropic response.² Equally important, nNOS was also involved in the development of heart failure, in which its expression was increased and accumulated in the sarcolemma.^{15,16} Because nNOS plays important roles in many aspects of cardiac physiology, the interaction of PMCA4b with nNOS is of particular interest. Importantly, PMCA4b is expressed in the heart,¹⁷ and we have recently demonstrated that PMCA4b-nNOS interaction also occurred in the cardiomyocyte.¹⁸

As a calcium pump, PMCA was previously assumed to contribute to diastolic Ca²⁺ extrusion.¹⁹ However, estimation of the contribution of PMCA to cardiomyocyte Ca²⁺ extrusion by use of inhibitors of other Ca²⁺ transporters (under the assumption that the remaining flux is due to PMCA) pointed to a very minor role of PMCA in diastolic Ca²⁺ extrusion.^{20,21} Indeed, the present data from transgenic animals support this notion, because no change in intracellular Ca²⁺ decay rate was observed in PMCA4b-overexpressing myocytes. A new concept that a classic ion pump can have a distinct function as signal transduction molecules has recently been proposed by independent groups, however. For example, it has been shown that Na⁺K⁺ATPase, a member of the P-type ATPase superfamily,²² transmits signals to the tyrosine kinase Src pathway in addition to its ion pumping function.²³ A number of arguments support the role for PMCA4 in molecular signaling: PMCA4 is localized to caveolae, which are rich in signaling molecules^{24,25}; it has been shown to modify growth²⁶ and differentiation,²⁷ to be involved in apoptosis,²⁸ and to modify responses to hypertrophic stimuli²⁵; and importantly, PMCA4 interacts with a number of signaling molecules, including nNOS.^{10,29–33} The present report dem-

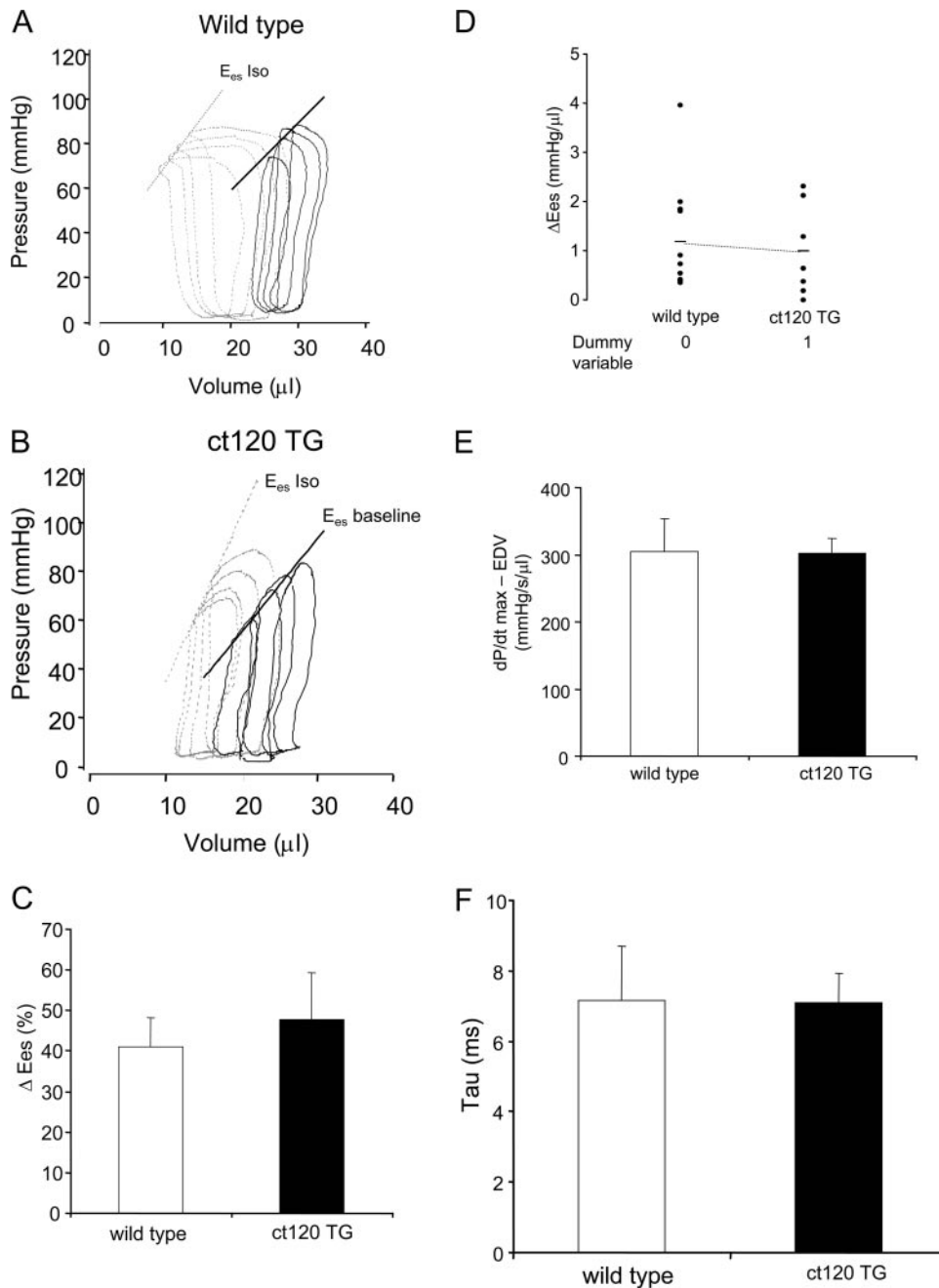


Figure 4. β -Adrenergic contractile response was not altered in ct120 transgenic(TG) mice. Representative pressure-volume loops and E_{es} at baseline (solid lines) and after isoproterenol induction (Iso; dashed lines) from (A) wild-type and (B) ct120 TG mice. C, Summary of the E_{es} change (ΔE_{es}). D, Regression analysis with dummy variables and (E) $dP/dt_{max} - EDV$ relationship in ct120 TG ($n=6$) and wild-type controls ($n=12$) showed no difference in the contractile response between ct120 TG and wild-type controls. F, The lusitropic response as indicated by relaxation time constant (τ) was not different between ct120 TG and wild-type controls.

onstrates the physiological relevance of the PMCA4b-nNOS interaction in the heart in vivo.

Transgenic overexpression of PMCA4b reduced the β -adrenergic inotropic response in vivo. Modulation of nNOS activity was likely the mechanism responsible for this phenotype, because (1) deletion of the C-terminal 120 amino acids of PMCA4, previously shown to mediate the interaction with nNOS,¹⁰ ablated this regulatory effect, and (2) treatment of wild-type and ct120 transgenic animals with the nNOS selective inhibitor L-nPA resulted in reduction of the β -adrenergic re-

sponse to levels comparable to that of PMCA4b transgenic mice, whereas treatment of PMCA4b transgenic animals with L-nPA did not reduce contractility in response to β -adrenergic stimulation. The potency and selectivity of nNOS inhibition by L-nPA is 149-fold relative to eNOS and 3158-fold relative to iNOS³⁴ which suggests that the response was not due to inhibition of other NOS isoforms. In addition, treatment of animals with N^G -nitro-L-arginine methyl ester, a nonspecific NOS inhibitor, in addition to L-nPA did not further alter the β -adrenergic response, which indicates that the response observed in wild-type

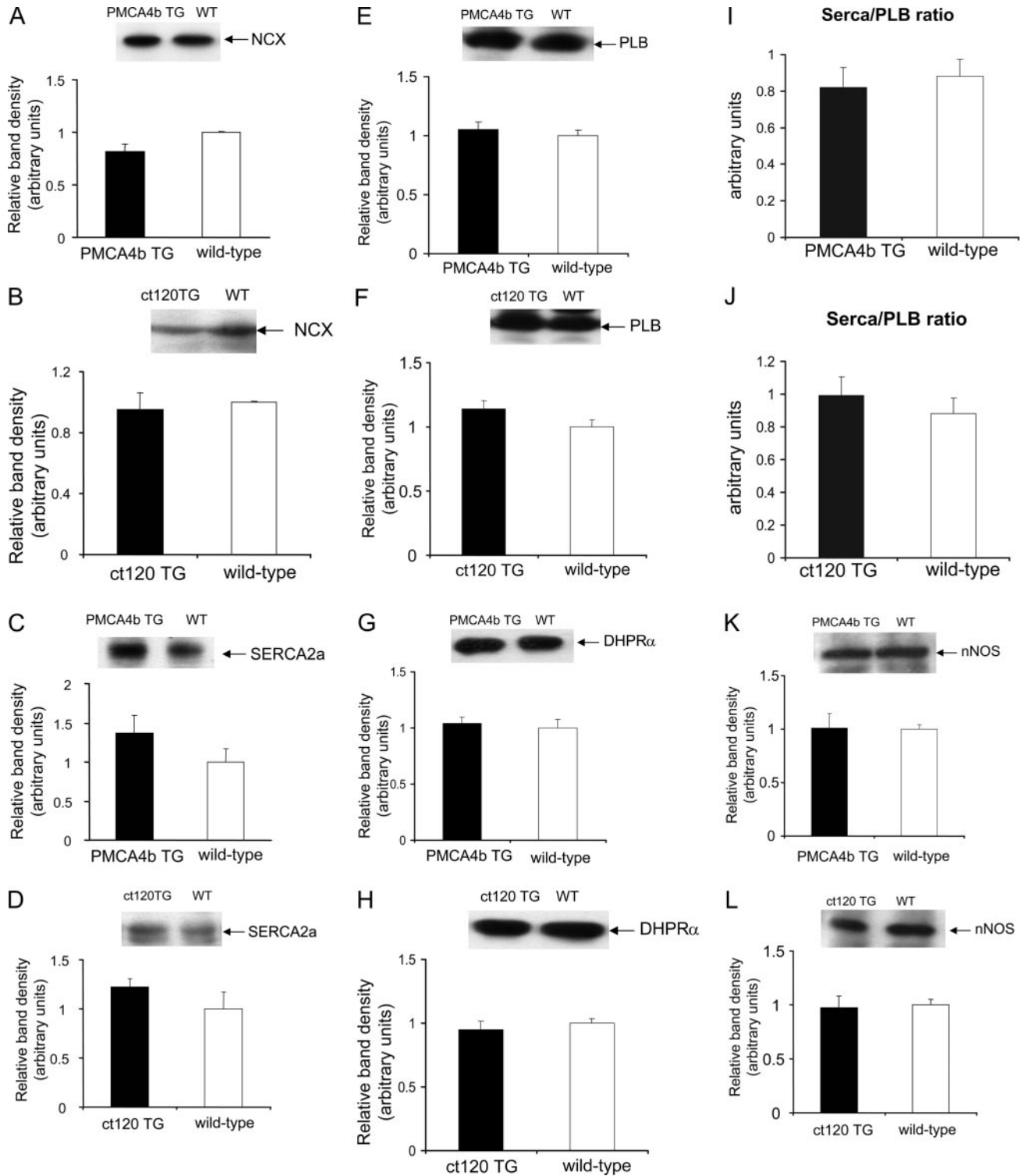


Figure 5. Expression of other calcium transporters in transgenic (TG) mice. Expression of Na⁺/Ca²⁺ exchanger (NCX; A, B), sarcoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a; C, D), phospholamban (PLB; E, F), dihydropyridine reductase-α (G, H), sarcoplasmic reticulum Ca²⁺-ATPase (SERCA)/phospholamban ratio (I, J), and nNOS (K, L) in the heart was detected by Western blot. No differences were found between PMCA4b TG mice and their wild-type (WT) littermates (A, C, E, G, I, K) or between ct120 TG mice and their wild-type controls (B, D, F, H, J, L) after normalization of the band density with GAPDH (n=5 in each group).

and ct120 transgenic animals after treatment with L-NPA was solely due to the inhibition of nNOS. These data are in line with previous findings in which mice with a genetic deletion of the nNOS gene displayed a reduced β-adrenergic contractile re-

sponse, whereas eNOS knockout mice have no reduction in inotropic response.^{2,35}

Expression of other major calcium transporters, as well as endogenous PMCA4 and nNOS, was not modified in these

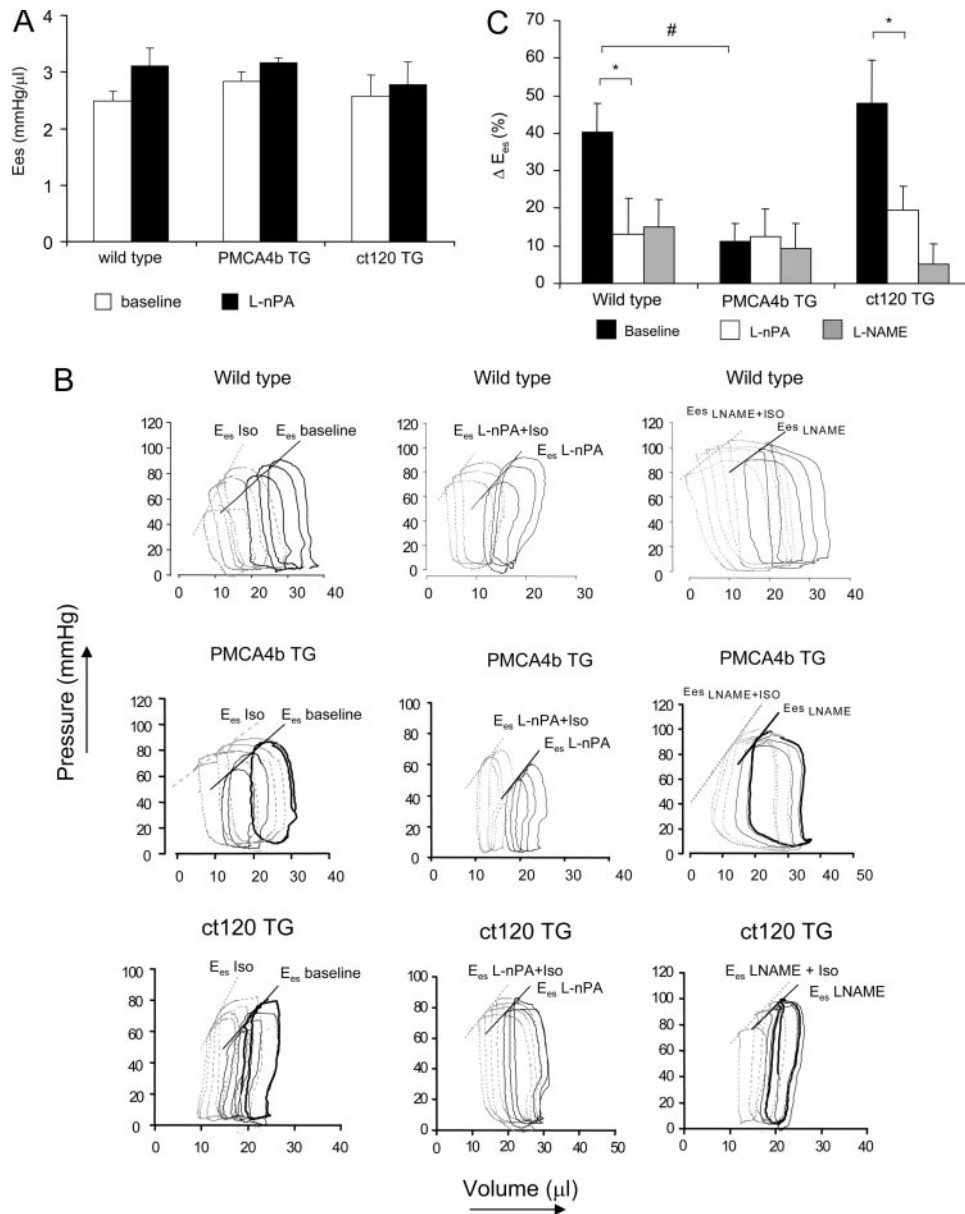


Figure 6. Effect of NOS inhibition on the β -adrenergic response in transgenic (TG) mice. **A**, Treatment with L-nPA did not significantly change baseline contractility, as indicated by E_{es} values. **B**, Representative pressure-volume loops and E_{es} after isoproterenol (Iso; 800 ng/kg body weight) induction in the presence of the selective nNOS inhibitor L-nPA (10 mg/kg body weight) and the nonselective NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; 50 mg/kg body weight) of wild-type, PMCA4b, and ct120 TG mice. **C**, Quantification of the β -adrenergic inotropic response indicated by the change of E_{es} . Statistical analysis with 2-factor ANOVA indicated that there was a significant effect of treatment with NOS inhibitors ($P < 0.05$) and an interaction between the 2 factors tested (genotype and treatment; $P < 0.05$), which suggests that the response to NOS inhibitors was different between animal groups. Contrast analysis and post hoc multiple comparison test indicated that there were significant differences in β -adrenergic inotropic response within wild-type and ct120 TG groups in the presence of L-nPA and N^G -nitro-L-arginine methyl ester (L-NAME) compared with baseline ($*P < 0.05$; $n = 5$). However, in PMCA4b TG mice, no significant reduction in β -adrenergic response was observed in the presence of L-nPA or N^G -nitro-L-arginine methyl ester ($n = 5$). In PMCA4b TG mice ($n = 5$), the baseline β -adrenergic inotropic response was attenuated compared with wild-type and ct120 TG mice. # $P < 0.05$.

transgenic lines. Because of the different affinities of the antibodies used, the absolute levels of PMCA4 and the nNOS protein levels cannot be compared directly; however, immunoprecipitation analysis demonstrated a higher level of nNOS interacting with PMCA4 in transgenic mice overexpressing PMCA4b. This suggests that the functional interaction between nNOS and PMCA4, and not modification of expression, is responsible for the observed phenotype. Because PMCA is

localized in sarcolemma, and nNOS has been found in both sarcoplasmic reticulum² and sarcolemma,³⁶ we speculate that the sarcolemma-nNOS and not the sarcoplasmic reticulum-nNOS is tightly regulated by the PMCA. In addition, another splice variant of nNOS (nNOS μ) is expressed in cardiomyocytes³⁷; however, there is no evidence whether nNOS μ is bound to the sarcolemma,³⁶ which suggests that PMCA4b interacts with the common splice variant of nNOS.

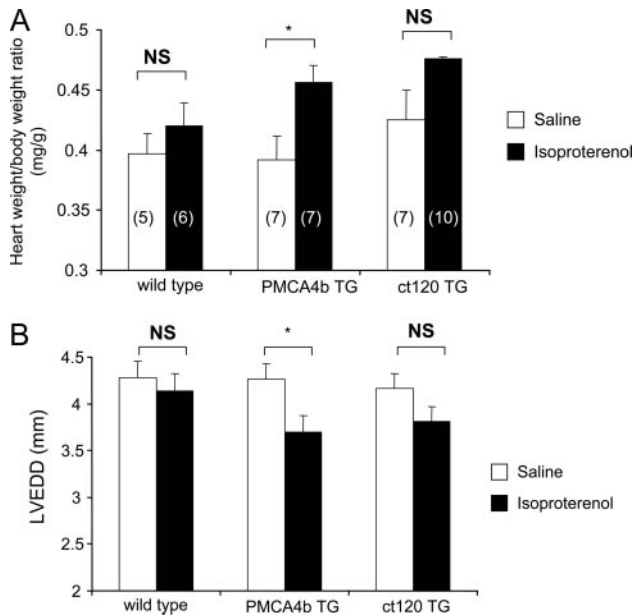


Figure 7. PMCA4b transgenic (TG) mice displayed increase hypertrophic response. A, Heart weight/body weight ratio in wild-type, PMCA4b TG, and ct120 TG mice after chronic stimulation with isoproterenol (10 mg/kg body weight per day for 7 days; solid bars) or injection with normal saline (open bars). Numbers in brackets denote number of animals in each group. Two-factor ANOVA showed that there was a significant effect of chronic isoproterenol treatment on cardiac size; however, analysis within groups indicated that a significant difference ($*P < 0.05$) between isoproterenol- and saline-treated animals was only found in the PMCA4b TG group. B, Left ventricular end-diastolic dimension (LVEDD) was significantly reduced in the isoproterenol-treated mice (2-factor ANOVA, $P < 0.05$); however, analysis within groups showed that a significant difference ($*P < 0.05$) between isoproterenol- and saline-treated animals was only found in the PMCA4b TG group.

Interestingly, despite modification of systolic function, diastolic function was not modified in PMCA4b transgenic mice. It is known that different sets of molecules regulate different steps during the excitation-contraction coupling cycle.³⁸ The present data imply that PMCA4b-mediated signaling through nNOS is more effective in systole. Because PMCA is believed to participate in calcium extrusion and is activated by calcium and calmodulin,³⁹ it is possible, therefore, that in systole, when intracellular calcium concentration is higher, the effect of nNOS inhibition is more substantial than in diastole, when intracellular calcium is low. Although in many cases, modification of systolic function will also affect diastolic function, the findings that baseline diastolic function in nNOS knockout animals was not significantly modified despite changes in systolic function support our hypothesis.^{2,4}

Given the finding that PMCA4b (via nNOS) regulates the adrenergic contractile response, the adrenergic system is likely one of the upstream signaling pathways of the PMCA4-nNOS complex, and therefore, inducing cardiac hypertrophy by long-term adrenergic stimulation was the logical approach to create a pathological model. As a first step to study this pathological effect, we used a low dose of isoproterenol to induce hypertrophy. As expected, a subtle increase of heart weight/body weight ratio was observed in wild-type mice; however, a more significant increase of heart weight/body weight ratio was found in

PMCA4b transgenic mice. It is possible that modulation of nNOS, at least in part, contributed to this phenotype, because ct120 transgenic mice displayed less hypertrophic response than PMCA4b transgenic mice. This finding is also in line with data from nNOS^{-/-} animals, in which mice lacking nNOS showed increased hypertrophy and remodeling.^{2,40}

This functional interaction between PMCA4b and nNOS has also been observed in vascular smooth muscle cells, which suggests a role for this mechanism in cardiovascular control. We and others have shown that transgenic overexpression of PMCA4b in mouse vascular smooth muscle cells resulted in an enhanced myogenic response and hence elevated blood pressure, due to the negative regulation of NO production from nNOS by PMCA.^{13,41}

In conclusion, the present data demonstrate that PMCA4b regulates the β -adrenergic contractile response via its interaction and modulation of nNOS activity. These results identify PMCA4b as a novel regulator of nNOS in the heart and strongly support an *in vivo* role of PMCA4b in signal transduction.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiac function largely depends on calcium transport that regulates 2 essential processes within the cardiomyocyte: contraction and molecular signaling. In a pathophysiological condition such as congestive heart failure, intracellular calcium homeostasis is impaired; therefore, a complete understanding of how the intracellular calcium is regulated in cardiomyocytes is a very important focus of research. In the present study, we investigated the function of plasma membrane calcium/calmodulin dependent ATPase 4b, one of the calcium transporters expressed in cardiomyocytes and located in the sarcolemma. Using genetically modified mice that overexpressed this molecule, we demonstrated that plasma membrane calcium/calmodulin dependent ATPase 4b regulates the β-adrenergic contractile response in vivo through interaction with and modulation of neuronal nitric oxide synthase without altering global intracellular calcium. Equally important, in response to long-term β-adrenergic stimulation, these animals developed increased hypertrophy, which suggests a possible role in pathophysiological conditions. Our present results provide the first in vivo evidence that plasma membrane calcium/calmodulin dependent ATPase 4b mediates calcium signaling independently of any direct action on the excitation-contraction process. Because modulation of NO signaling has increasingly been known to have beneficial effects in heart failure, these findings establish plasma membrane calcium/calmodulin dependent ATPase as a potential novel target for the development of new treatments for heart failure.